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Spironolactone suppresses inflammation and prevents L-NAME–induced renal injury in rats

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Chronic inhibition of nitric oxide synthase by N^{ω}-nitro-L-arginine methyl ester (L-NAME) causes progressive renal injury with systemic hypertension and interstitial macrophage infiltration. We have previously shown that there is local activation of the renin–angiotensin–aldosterone system in the renal cortex as a major pathogenic feature of macrophage infiltration. In this study, we measured the effects of the aldosterone antagonist, spironolactone, on renal injury in L-NAME-treated male Wistar rats. After 12 weeks of L-NAME-treatment, rats had increased systolic blood pressure, urinary protein excretion, and serum creatinine and histological analysis showed glomerulosclerosis, interstitial fibrosis, and macrophage infiltration. Treatment with spironolactone significantly prevented these renal changes, whereas treatment with hydralazine had no effect. The cortical expression of osteopontin was significantly elevated in L-NAME-treated rats, and expression of its mRNA significantly correlated with the number of infiltrating macrophages and degree of interstitial fibrosis. Spironolactone treatment markedly suppressed osteopontin expression. Our results suggest that reduced nitric oxide bioavailability caused renal inflammation and fibrosis through an aldosterone receptor–dependent mechanism associated with osteopontin expression independent of its systemic hemodynamic effects.

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KEYWORDS: nitric oxide; aldosterone; osteopontin; renin–angiotensin system; interstitial fibrosis

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Angiotensin II (Ang II) is a major player in the progression of many types of renal diseases.[1] Angiotensin-converting enzyme inhibitor (ACEi) and Ang II type 1 (AT1) receptor antagonist are clinically effective in reducing proteinuria and in slowing the progression of proteinuric renal disease,[2] and are regarded as first-choice therapy for patients with chronic kidney disease. Recent experimental evidence implicated both aldosterone and Ang II in chronic kidney disease in various animal models of renal injury.[3–8] ACEi and AT1 receptor antagonist can also mitigate aldosterone levels, although the suppression is usually not complete.[9] Bianchi *et al.*[10] demonstrated that aldosterone antagonist, spironolactone (Spi), reduced proteinuria and retarded the progression of chronic kidney disease in patients already treated with ACEi and/or AT1 receptor antagonist. However, the mechanism underlying this beneficial effect of aldosterone blockade remains unclear.

N^{ω}-nitro-L-arginine methyl ester (L-NAME) is an inhibitor of nitric oxide synthase (NOS), which is known to induce systemic hypertension in association with progressive renal tissue injury.[11–14] Rats treated with L-NAME are an established model of progressive chronic kidney disease with systemic hypertension. These animals exhibit massive infiltration of macrophages in renal cortex interstitium.[14] There is increasing evidence that tubulointerstitial inflammation, especially macrophage infiltration, leads to interstitial fibrosis.[15] Previous studies from our laboratory in L-NAME-treated rats demonstrated local activation of the renin–angiotensin–aldosterone system in the renal cortex as a major pathogenic feature of macrophage infiltration. Treatment with ACEi or AT1 receptor antagonist suppressed macrophage infiltration and conferred renoprotection.[16,17] Ang II is a major mediator of inflammation in this model. Several recent studies also demonstrated that aldosterone promotes inflammation in kidney disease.[3,18]

Osteopontin (OPN) is a potent chemotactic and adhesive factor for macrophages.[19,20] Upregulation of OPN expression in renal tubules is strongly associated with macrophage infiltration subsequent to tubulointerstitial injury both in experimental models[21] and in patients with kidney disease.[22] Furthermore, Blasi *et al.*[3] demonstrated a role for OPN in the renal inflammatory response following aldosterone/salt

treatment via macrophage recruitment, whereas the selective aldosterone blocker, eplerenone, suppressed both OPN expression and inflammatory changes.

We hypothesized that reduced NO bioactivity induces renal inflammation and renal fibrosis through an aldosterone receptor-dependent mechanism, especially in association with OPN expression. To test this hypothesis, we examined the effects of aldosterone antagonist Spi in the L-NAME-treated rat model.

RESULTS

Clinical parameters

Figure 1 shows serial changes in blood pressure and proteinuria in the four rat groups. Systolic blood pressure (SBP) did not increase in control rats over the 12-week study (final measurement 146.0 ± 3.7 mm Hg, mean ± s.e.m.). L-NAME treatment induced a progressive increase in SBP over time, resulting in severe hypertension (final measurement 239.5 ± 2.2 mm Hg). Rats treated with L-NAME + Spi and L-NAME + hydralazine (Hyd) also showed an increase in SBP. However, these increases were not as high as in the L-NAME-treated rats and did not change throughout the study period (final measurement 196.1 ± 4.8 and 211.1 ± 3.8 mm Hg, respectively). Twelve weeks after initiation of treatment, urinary protein was markedly elevated in the L-NAME (162.6 ± 30.1 mg per 24 h) and L-NAME + Hyd (219.6 ± 46.5 mg per 24 h) groups, compared with L-NAME-Spi-treated (43.8 ± 16.8 mg per 24 h) and control (9.0 ± 1.0 mg per 24 h) rats.

Table 1 lists the other physiological and biochemical changes induced in each group. Body weight after 12 weeks was significantly lower in L-NAME and L-NAME + Hyd groups compared with control rats. Serum creatinine after 12 weeks was higher in both L-NAME and L-NAME + Hyd groups compared with other groups. Spi treatment did not induce a significant rise in serum potassium level compared with the control. Finally, plasma Ang II and aldosterone levels were significantly higher in rats treated with L-NAME compared with the control (231.6 ± 42.6 versus 47.1 ± 4.3 pg/ml, $P<0.05$, 6562.9 ± 1612.5 versus 129.3 ± 8.9 pg/ml, $P<0.05$). Spironolactone prevented the elevation of plasma Ang II and aldosterone levels.

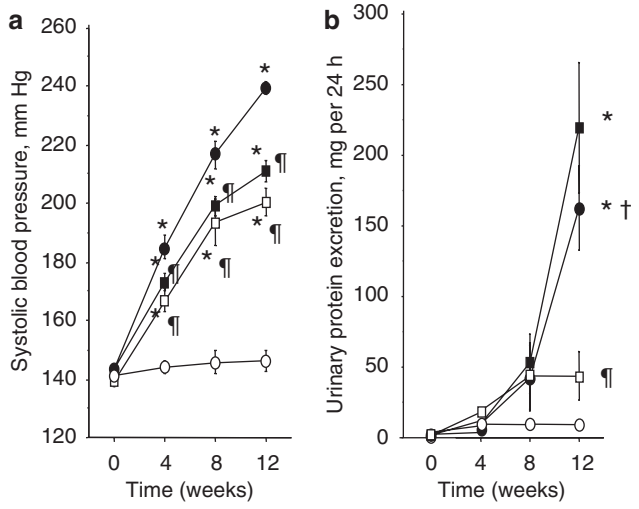

Figure 1 | Changes in blood pressure and proteinuria. (**a**) Systolic blood pressure measured by the tail-cuff method and (**b**) proteinuria. Values are mean ± s.e.m. (○) Control, (●) L-NAME-treated, (□) L-NAME + Spi-treated, and (■) L-NAME + Hyd-treated. *$P<0.05$ versus control rats, ¶$P<0.05$ versus L-NAME-treated rats, †$P<0.05$ versus L-NAME + Spi-treated rats.

Effects of Spi on glomerulosclerosis and interstitial fibrosis

Figures 2 and 3 show the typical glomerular and interstitial pathology in the four experimental groups at the end of the study period. Control rats were histologically normal (Figures 2a and 3a). L-NAME-treated rats showed glomerular damage characterized by arteriolar hyaline change and global or segmental sclerosis (Figure 2b). L-NAME-treated rats also showed renal tubular atrophy of the interstitium, as well as inflammatory cell infiltration and interstitial fibrosis (Figure 3b). Semiquantitative analysis revealed significantly severe glomerulosclerosis in the L-NAME group compared with control rats (1.6 ± 0.2 versus 0.3 ± 0.1, $P<0.05$; Figure 2e), and this was prevented by Spi treatment (0.5 ± 0.1 versus 1.6 ± 0.2, $P<0.05$; Figure 2e). Interstitial fibrosis was prominent in the cortical regions of L-NAME-treated rats compared with control rats (9.9 ± 0.5 versus 3.2 ± 0.3%, $P<0.05$; Figure 3e), and this fibrotic change was also attenuated in the L-NAME + Spi group (3.7 ± 0.3 versus 9.9 ± 0.5%, $P<0.05$; Figure 3e). In the L-NAME + Hyd group, the glomerulosclerosis and interstitial fibrosis were not ameliorated despite the antihypertensive effect (Figures 2e and 3e).

Effects of Spi on OPN mRNA and protein expression

The expression of OPN mRNA in renal cortex was significantly higher in the L-NAME group than in controls, and this upregulation was suppressed by Spi treatment (Figure 4a). Immunohistochemical analysis showed localization of OPN protein expression to medullary tubular cells in control rats, whereas most cortical structures were OPN-negative (Figure 4b). OPN protein expression was markedly elevated in L-NAME-treated rats, localizing to the cytoplasm of tubular epithelial cells (Figure 4c). Strong OPN expression was noted in areas with severe tissue damage such as atrophic tubules, although some OPN immunoreactivity was also observed in mildly injured tubules. This upregulation of OPN protein expression was markedly suppressed in the L-NAME + Spi group (Figure 4d).

Effects of Spi on macrophage infiltration

Macrophages, detected by immunohistochemical staining for ED-1, were rarely observed within the tubular interstitium of control rats (Figure 5a), whereas L-NAME rats showed significant macrophage infiltration (Figures 5b and e, 20.3 ± 2.3 cells per field in L-NAME group versus 0.4 ± 0.1 cells per field in controls, $P<0.05$). Macrophage

Table 1 | Clinical parameters of the four experimental groups

	Control	L-NAME	L-NAME+Spironolactone	L-NAME+Hydralazine
Body weight (g)				
Before	368 ± 3	363 ± 3	362 ± 5	364 ± 3
4 week	473 ± 11	453 ± 8	450 ± 9	456 ± 8
8 week	538 ± 12	501 ± 11*	516 ± 5	505 ± 10
12 week	577 ± 11	501 ± 20*	534 ± 17	491 ± 18*
Serum creatinine (mg/100 ml)				
12 week (mEq/l)	0.26 ± 0.02	0.77 ± 0.15*	0.36 ± 0.02¶	0.74 ± 0.14*†
Serum potassium (pg/ml)				
12 week	4.6 ± 0.2	5.2 ± 0.3	4.9 ± 0.2	4.9 ± 0.3
Plasma aldosterone (pg/ml)				
12 week	129.3 ± 8.9	6562.9 ± 1612.5*	1447.3 ± 536.6¶	4570.6 ± 1403.3*
Plasma angiotensin II (pg/ml)				
12 week	47.1 ± 4.3	231.6 ± 42.6*	56.1 ± 11.3¶	141.9 ± 48.0

L-NAME, N^{ω}-nitro-L-arginine methyl ester.
Data are expressed as mean ± s.e.m.
*$P<0.05$ versus control rats, ¶$P<0.05$ versus L-NAME treated rats, †$P<0.05$ versus L-NAME/spironolactone treated rats.

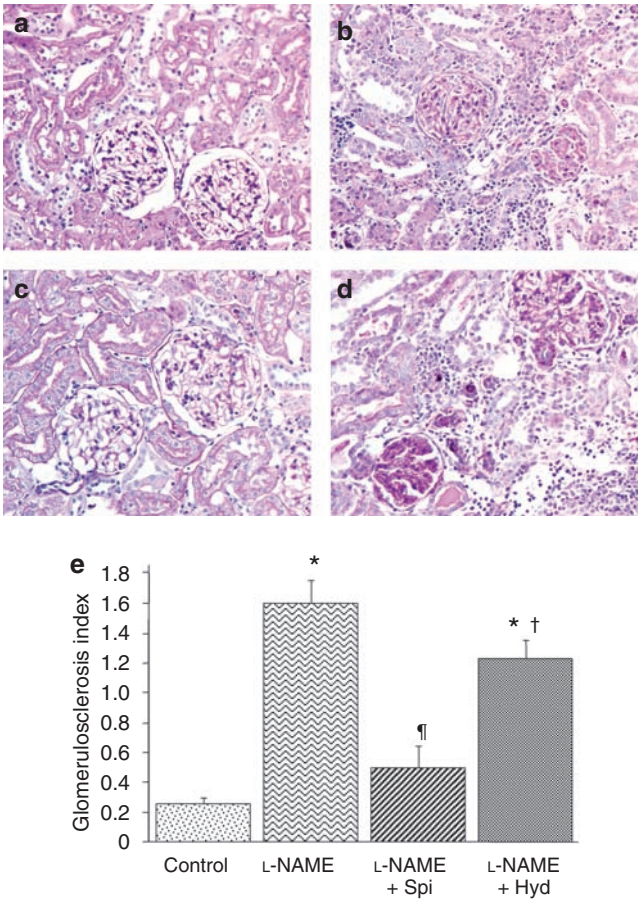

Figure 2 | Representative photomicrographs of renal tissue and semiquantitative analysis of glomerulosclerosis indices. Periodic-acid Schiff staining of sections from (**a**) control rats, (**b**) L-NAME-treated rats after 12 weeks, (**c**) L-NAME + Spi-treated rats after 12 weeks, (**d**) L-NAME + Hyd-treated rats after 12 weeks, and (**e**) glomerulosclerosis in each group; original magnification × 200. Values are mean ± s.e.m. *$P<0.05$ versus control rats, ¶$P<0.05$ versus L-NAME-treated rats, †$P<0.05$ versus L-NAME + Spi-treated rats.

infiltration was markedly suppressed by Spi treatment (Figure 5c and e, 20.3 ± 2.3 versus 1.1 ± 0.5 cells per field, $P<0.05$).

Correlation between OPN expression and number of ED-1-positive cells and interstitial fibrosis

We next analyzed the correlation between OPN mRNA expression and macrophage infiltration and interstitial fibrosis in the model rats (Figure 6). The degree of OPN mRNA expression correlated well with both the number of ED-1-positive cells ($r=0.725$ and $P<0.05$) and the level of interstitial fibrosis ($r=0.753$ and $P<0.05$) in all groups.

Effects of Spi on transforming growth factor-β1 and α-smooth muscle actin protein expression

Figure 7 shows representative immunostaining for transforming growth factor (TGF)-β1 (Figure 7a–d) and α-smooth muscle actin (α-SMA) (Figure 7e–h) in kidney sections from all groups. Positive immunostaining for TGF-β1 and α-SMA was detected only occasionally in the control rats (Figure 7a and e). L-NAME-treated rats showed immunoreactivity for TGF-β1 in the cytoplasm of tubular epithelial cells (Figure 7b) and α-SMA expression in interstitial areas (Figure 7f). Spi treatment suppressed TGF-β1 and α-SMA expression (Figure 7c and g).

DISCUSSION

This study on L-NAME–induced renal damage demonstrated that aldosterone blockade by Spi suppresses urinary protein excretion and inhibits both glomerulosclerosis and fibrosis associated with attenuation of the renal inflammatory process. The results indicated that aldosterone contributes to renal inflammation in this rat model of chronic kidney disease with systemic hypertension.

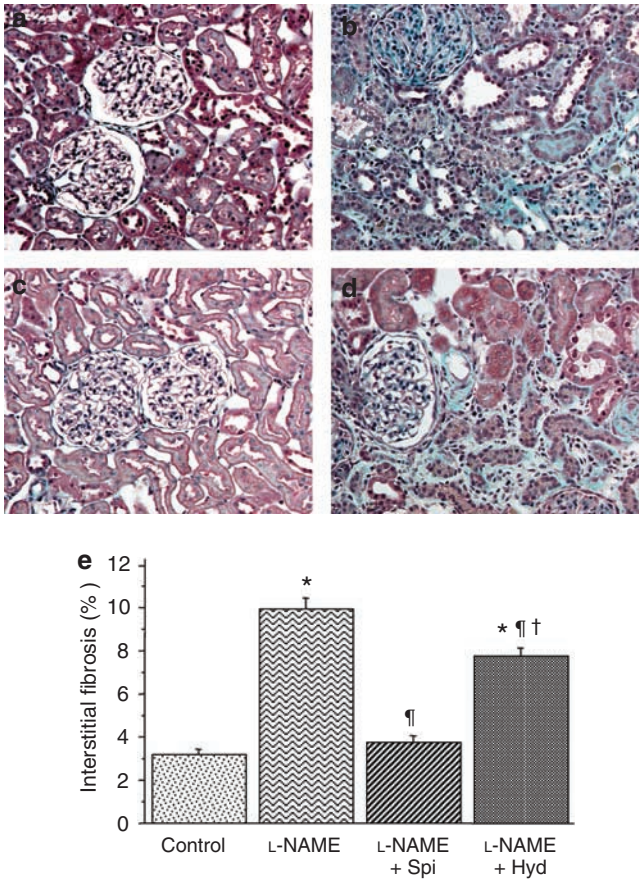

Figure 3 | Representative photomicrographs of renal tissue and semiquantitative analysis of interstitial fibrosis. Masson trichrome staining of sections from (**a**) control rats, (**b**) L-NAME-treated rats after 12 weeks, (**c**) L-NAME + Spi-treated rats after 12 weeks, (**d**) L-NAME + Hyd-treated rats after 12 weeks, and (**e**) percentage of interstitial fibrosis in each group; original magnification × 200. Values are mean ± s.e.m. *$P<0.05$ versus control rats, ¶$P<0.05$ versus L-NAME-treated rats, †$P<0.05$ versus L-NAME + Spi-treated rats.

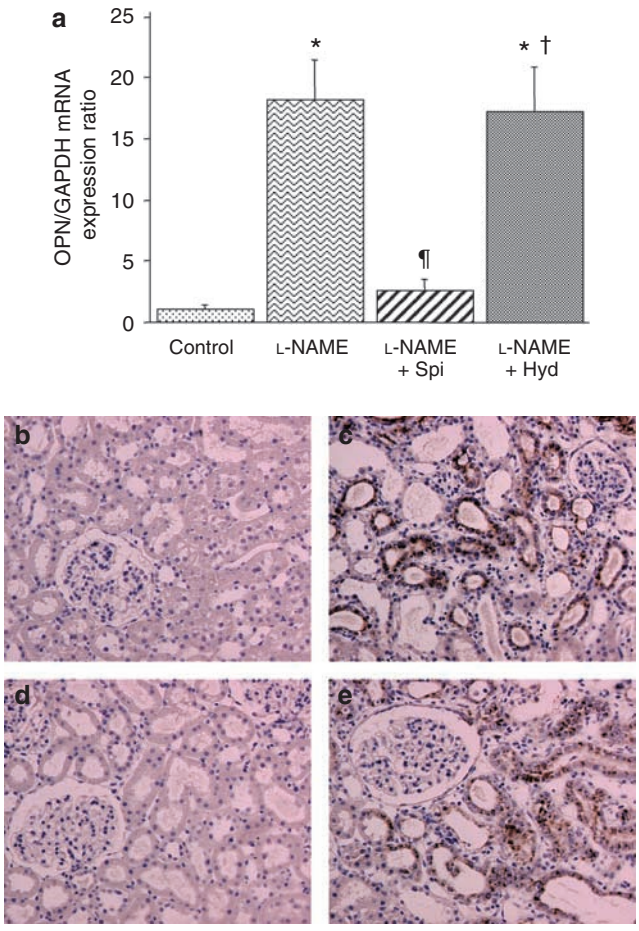

Figure 4 | Renal expression of OPN mRNA and immunohistochemistry for OPN protein. (**a**) Renal cortical OPN mRNA was measured by real-time RT-PCR. Immunohistochemistry of OPN protein in (**b**) control rats, (**c**) L-NAME-treated rats after 12 weeks, (**d**) L-NAME + Spi-treated rats after 12 weeks, and (**e**) L-NAME + Hyd-treated rats after 12 weeks. Positive staining for OPN was observed mainly in the flattened epithelial cells of the dilated tubular lumen and in cell debris for L-NAME- and L-NAME + Hyd-treated rats. Values are mean ± s.e.m. *$P<0.05$ versus control rats, ¶$P<0.05$ versus L-NAME-treated rats, †$P<0.05$ versus L-NAME + Spi-treated rats; original magnification × 200.

Similar to previous reports,[16,17] L-NAME-treated rats in this study developed significant hypertension, proteinuria, severe renal inflammation marked by a large number of infiltrating macrophages, and renal fibrosis. Prolonged NOS inhibition by L-NAME activates both systemic and local renal Ang II, regarded as a major mechanism of renal injury in this animal model.[11,12,16,23,24] Our previous study demonstrated that ACEi and AT1 receptor antagonist confer pathophysiological renoprotection in L-NAME-treated rats.[16,17] However, Ang II also stimulates the synthesis of aldosterone. Furthermore, Klar *et al.*[25] demonstrated that aldosterone exerts a direct positive effect on renin gene expression in renal juxtaglomerular cells. It has been demonstrated also that aldosterone upregulates angiotensin-converting enzyme mRNA expression and its enzymatic activity in rat endothelial cells.[26] These effects are blocked by Spi. In addition, previous experiments demonstrated that chronic NO blockade by L-NAME induces adrenal aldosterone synthesis.[27,28] In the present study, plasma Ang II and plasma aldosterone concentrations were increased in L-NAME-treated rats. The results showed that inhibition of aldosterone by Spi also attenuated proteinuria and renal inflammation in L-NAME-treated rats. Spi might inhibit the positive feedback by suppressing the effects of aldosterone as well as the elevated concentrations of plasma Ang II and aldosterone in L-NAME-treated rats.

In addition to renal protection, Spi induced a significant decrease in blood pressure, thereby possibly augmenting the attenuation of renal damage and inflammation. However, reducing the hypertension using Hyd had a much smaller effect on the prevention of renal damage than Spi treatment. Of note, there was no significant difference in SBP between L-NAME-Spi and L-NAME + Hyd groups. These findings suggest that aldosterone participates in the L-NAME-induced renal damage independently of systemic hemodynamic effects.

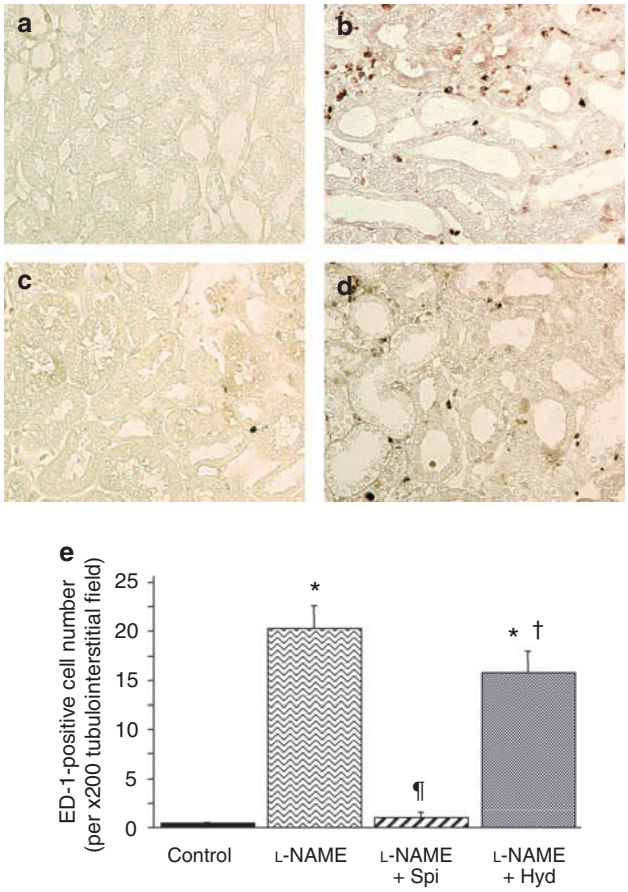

Figure 5 | Representative images of immunohistochemistry for ED-1 and quantification of ED-1-positive cells within the tubular interstitium. (**a**) Control rats, (**b**) L-NAME-treated rats, (**c**) L-NAME + Spi-treated rats, (**d**) L-NAME + Hyd-treated rats, and (**e**) ED-1-positive cell number in each group. L-NAME-treated rats showed focal accumulation of ED-1-positive cells within the tubular interstitium compared with control rats, especially in injured areas. Spi significantly suppressed the number of ED-1-positive cells. Values are mean ± s.e.m. $^{*}P<0.05$ versus control rats, $^{¶}P<0.05$ versus L-NAME-treated rats, $^{†}P<0.05$ versus L-NAME + Spi-treated rats; original magnification × 200.

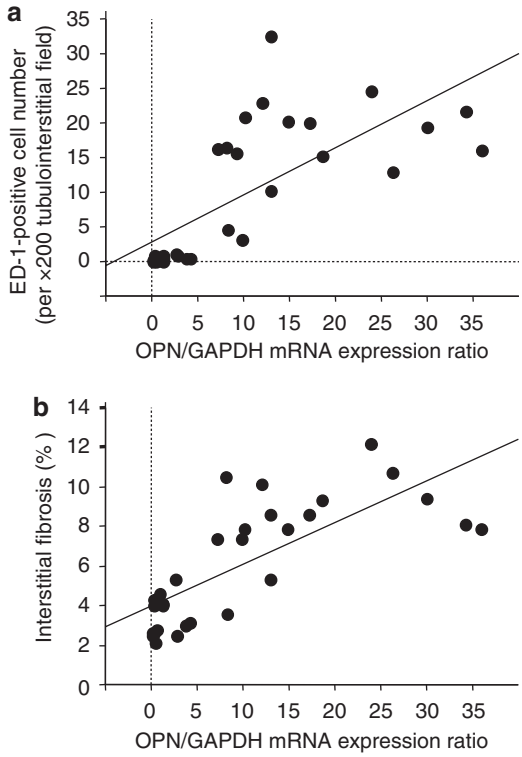

Figure 6 | Correlation of OPN mRNA expression with macrophage accumulation and tubulointerstitial injury. OPN mRNA expression correlated significantly with the number of (**a**) ED-1-positive cells and (**b**) interstitial fibrosis. Data were analyzed by Pearson's single correlation coefficient; in **a**, $r=0.725$ and $P<0.05$ and in **b**, $r=0.753$ and $P<0.05$.

Previous studies, *in vivo*[27,28] and *in vitro*,[29–32] indicated that endogenous NO directly reduces adrenal aldosterone synthesis and that NO inhibition induces adrenal aldosterone synthesis. Nithipatikom *et al.*[31] demonstrated that long-term administration of NO inhibits aldosterone synthesis in zona glomerulosa cells by downregulation of the expression of AT1 receptors. Usui *et al.*[27] demonstrated that inhibition of NO synthesis by L-NAME increases AT1 receptor expression and induces adrenal aldosterone synthesis. Moreover, Tsukamoto *et al.*[28] demonstrated that chronic inhibition of NO synthesis increases the expression of CYP11B2 mRNA in adrenal glands of rats and induces adrenal aldosterone synthesis. On the other hand, aldosterone might suppress NOS activity in L-NAME-treated rats. Pechanova *et al.*[33] demonstrated that Spi increased endothelial NOS protein expression and prevented the depression of NOS activity in L-NAME-treated rats. Thus, in addition to the direct effect of aldosterone blockade, Spi might prevent a vicious cycle between activation of renin–angiotensin–aldosterone system and reduced NO bioavailability in the L-NAME-treated rat model.

In some experimental models of kidney disease related to aldosterone, such as aldosterone/salt-treated rats model,[3] a salt load was needed to induce renal injury. Salt loading is reported to enhance reactive oxygen species generation. Kitiyakara *et al.*[34] demonstrated that high salt intake induces oxidative stress, which is associated with activated renal-reduced nicotinamideadenine dinucleotide phosphate oxidase, and decreases intracellular superoxide dismutase. In our experiments, all animals were fed normal-salt diet (0.45% Na) during the study. We thought that L-NAME, in addition to activation of renin–angiotensin–aldosterone system, decreased NO level and increased oxidative stress and induced renal injury without salt.

The L-NAME-treated rat model is characterized by massive interstitial infiltration of macrophages.[14,17] Our previous study associated this macrophage infiltration with Ang II.[17] Considerable recent evidence also indicates that aldosterone induces vascular inflammation via activation of proinflammatory cytokines,[35,36] and some studies suggested that such activation in the kidney leads to renal inflammation.[3,18] The present study confirmed that blocking aldosterone using Spi reduces macrophage infiltration.

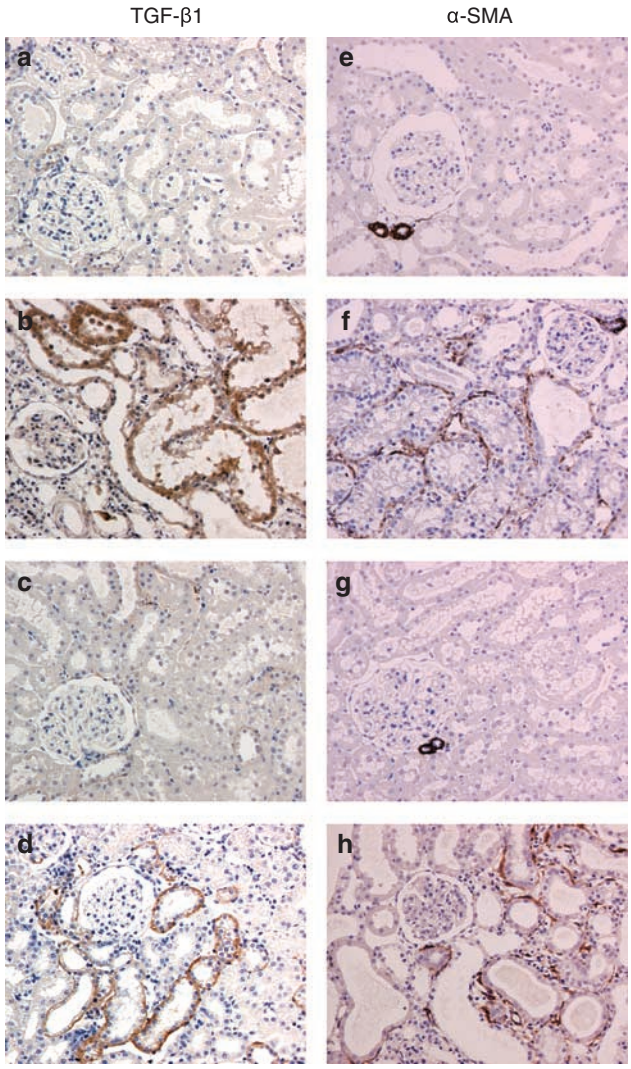

Figure 7 | Representative images of immunohistochemistry for TGF-β1 (a–d) and α-SMA (e–g) in renal cortex. (**a**, **e**) Control rats, (**b**, **f**) L-NAME-treated rats, (**c**, **g**) L-NAME + Spi-treated rats, and (**d**, **h**) L-NAME + Hyd-treated rats. TGF-β1 and α-SMA protein was virtually absent from the cortical tubules and interstitium of control rats, but was strongly expressed in the cortex of L-NAME-treated rats. Spi treatment reversed the increase in TGF-β1 and α-SMA expression; original magnification × 100.

There is considerable experimental evidence that OPN is closely related to macrophage infiltration. OPN is expressed in some renal distal tubules in normal rat kidneys. In several models of glomerular and tubulointerstitial injury, tubular OPN expression is markedly increased.[37,38] Overexpression of OPN in the tubules correlated with the degree and site of interstitial macrophage infiltration and development of interstitial fibrosis.[37,38] This was confirmed in OPN-gene knockout mice,[39] and in animals treated with antisense probes or anti-OPN antibody,[40] where significant reduction in macrophage infiltration and renal fibrosis was demonstrated. The present study showed upregulation of OPN at both the mRNA and protein levels in L-NAME-treated rats in parallel with increased macrophage numbers, as indicated by ED-1-positive cells. This OPN upregulation was suppressed significantly by Spi, concomitant with a decrease in macrophages. The present study thus implicates aldosterone in the upregulation of OPN in the L-NAME rat model, and demonstrates a renoprotective role for Spi via the down-regulation of OPN. The mechanism by which aldosterone stimulates OPN expression in this model is not fully understood. Although aldosterone could act indirectly, some studies showed that treatment with aldosterone induced OPN expression directly, both *in vivo*[3] and *in vitro*.[41] Recently, Irita *et al.*[41] demonstrated that aldosterone induced mineralocorticoid receptor-mediated OPN expression through activation of nuclear factor-κB and suggested that aldosterone is important in renal fibrosis through the induction of OPN in renal fibroblasts. Nuclear factor-κB activation has been already demonstrated in L-NAME-treated rats.[42] We suspect that OPN is upregulated by aldosterone through activation of nuclear factor-κB in the L-NAME-treated rat model. Our results demonstrated that the aldosterone antagonist Spi inhibited OPN mRNA upregulation and reduced renal inflammation. These findings suggest that aldosterone could induce renal inflammation through a mineralocorticoid receptor-dependent mechanism.

Aldosterone could cause renal injury in L-NAME-treated rats by pathways other than inflammation. Aldosterone injection in normal rats provoked renal TGF-β1 synthesis,[43] and TGF-β1 is a major fibrogenic cytokine in both interstitial fibrosis and glomerulosclerosis.[44] Many experiments also suggested that fibroblasts, particularly myofibroblasts, distinguished by α-SMA expression, are key cells in the development of renal interstitial fibrosis.[45,46] In this study, we observed many TGF-β1- and α-SMA-positive cells in L-NAME-treated rats. Spi treatment reduced the expression of both proteins. TGF-β1 is also a key mediator of tubular epithelial cell transdifferentiation into α-SMA-positive myofibroblasts.[47] In addition, Zhang *et al.*[48] recently demonstrated that aldosterone induces epithelial–mesenchymal transition in renal epithelial tubular cells. Aldosterone might therefore cause renal injury in the L-NAME rat model, in part, through myofibroblast trans differentiation.

In conclusion, our study demonstrated that reduced NO bioactivity induces renal inflammation and renal fibrosis through an aldosterone receptor-dependent mechanism. These inflammatory and fibrotic effects may result from upregulation of OPN independent of systemic hemodynamic effects.

MATERIALS AND METHODS

Animals

Experiments were conducted on 10-week-old male Wistar rats, weighing 320–350 g. Rats were treated according to protocols approved by the Kyushu University Animal Care Committees at the Center Animal Care facility. These experiments were reviewed and approved by the Committee on Ethics of Animal Experiments,

Kyushu University Faculty of Medicine, and were conducted according to the Guidelines for Animal Experiments of the Kyushu University Faculty of Medicine. The animals were housed in a temperature-controlled room with a 12-h light–dark cycle. Animals were allowed 1 week to adjust after arrival and allowed free access to food and water during the entire study. All animals were fed normal-salt diet (0.45% Na; CLEA Japan, Tokyo, Japan) throughout the study. Rats were killed by intraperitoneal injection of 50 mg/kg sodium pentobarbital (Dinabot Co., Osaka, Japan).

Experimental design

The rats were separated randomly into four groups and treated for 12 weeks. The control group was given normal drinking water ($n=8$). The disease control group received drinking water containing L-NAME (0.5 mg/ml, $n=8$; Sigma, St Louis, MO, USA). At this concentration, the daily intake of L-NAME was 50 mg/kg/day. The third group received L-NAME in the drinking water and the aldosterone receptor blocker Spi (100 mg/kg/day, $n=8$; Sigma), incorporated into the rodent diet. The fourth group received L-NAME with Hyd (6 mg/kg/day, $n=8$; Wako Pure Chemical Industries, Osaka, Japan) in the drinking water. The actual volumes of consumed chow and drinking water were measured separately every 4 weeks while the rats were in metabolic cages (CLEA Japan). We also confirmed that individual values for food and water intake were similar among the groups. At the end of the study, blood samples were collected by cannulation of the abdominal aorta, and stored at $-80\,^{\circ}C$. The kidneys were perfused with cold phosphate-buffered saline (pH 7.4) and then excised. Renal capsules from the left kidney were gently removed and the cortex removed using scissors. The cortical tissue samples were snap-frozen in liquid nitrogen and stored at $-80\,^{\circ}C$ for molecular analysis. Tissue from the right kidney was fixed in 4% paraformaldehyde (Wako Pure Chemical Industries) for later use.

Clinical parameters

Rat body weight and SBP were measured every 4 weeks during the study. Blood pressure was monitored in the conscious state using the tail-cuff method (BP-monitor MK-2000; Muromachi Kikai, Tokyo, Japan). Twenty-four-hour urine samples were collected every 4 weeks in metabolic cages during food and water intake measurement. Urinary protein excretion was measured by the pyrogallol sulfonphthalein method. Serum creatinine as well as blood urea nitrogen and potassium were analyzed using a Hitachi 7170 autoanalyzer (Hitachi, Tokyo, Japan). Finally, plasma Ang II and aldosterone levels were analyzed by radioimmunoassay.

Histochemical staining and quantification

The kidney tissues fixed in 4% paraformaldehyde were embedded in paraffin for light microscopy. Two-micron sections were stained with periodic acid-Schiff reagent and Masson trichrome. Glomerulosclerosis was assessed on periodic acid-Schiff stained sections. Glomerular mesangial matrix was assessed semiquantitatively on 100 glomeruli selected at random, and the degree of expansion determined using the method of Raij *et al.*[49] The percentage of each glomerulus occupied by mesangial matrix was estimated and scored as follows: 0, no lesion; 1+, 1–25%; 2+, 26–50%; 3+, 51–75%; and 4+, 76–100%. The number of glomeruli showing no lesion was set to $n0$; similarly, 1+ to $n1$, 2+ to $n2$, 3+ to $n3$, and 4+ to $n4$. The sclerosis index for 100 glomeruli was calculated by the following formula: glomerulosclerosis index $=((0\times n0+1\times n1+2\times n2+3\times n3+4\times n4)/100)\times 100$. Interstitial fibrosis was assessed on the Masson trichrome-stained sections. A representative section of whole cortex was analyzed by the point-counting technique to obtain the relative interstitial volume,[50] using a 121-point (100 square) eyepiece micrometer. Renal interstitial injury was estimated by counting the relative interstitial volume, using a minimum of five sections (605 points) randomly selected in all groups.

Immunohistochemistry and quantification

Kidney sections were immunostained for ED-1, OPN, TGF-β1, and α-SMA as described previously.[51] Briefly, 2-μm-thick sections were deparaffinized and then treated to inactivate endogenous peroxidase by incubation in 0.3% H_2O_2 in methanol for 30 min. The sections were then preincubated with 5% skim milk to decrease nonspecific binding and incubated overnight at 4 °C with anti-ED-1 (1:2000; Chemicon International, Temecula, CA, USA), anti-OPN (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-TGF-β1 (1:100; Santa Cruz Biotechnology), or anti-α-SMA (1:5; Nichirei, Tokyo, Japan) antibody. After extensive washing, the sections were incubated with biotinylated secondary antibodies for 1 h at room temperature followed by horseradish peroxidase conjugated streptavidin (100 μg/ml; Nichirei) for 30 min. The horseradish peroxidase was visualized by reaction with 3,3′-diaminobenzidine tetrahydrochloride (DAB; Nichirei) and H_2O_2. ED-1-positive cells in the cortex were counted to quantify infiltrating interstitial macrophages. The numbers from 20 consecutive high-power fields (× 200) were averaged (cells per high-power field).

RNA isolation and real-time PCR

Total RNA was isolated from the renal cortical tissue using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the protocol provided by the manufacturer. One-step real-time reverse transcription (RT)-PCR was conducted using the LightCycler system (Roche Diagnostics, Mannheim, Germany) and 5 ng of total RNA. The primers and probes for rat OPN and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were as follows: OPN forward primer, 5′-GGAGTATAAGCAGAGGGCCA-3′; OPN reverse primer, 5′-CGCCTGACTGTCGATAGCAT-3′, GAPDH forward primer, 5′-TGAACGGGAAGCTCACTGG-3′; GAPDH reverse primer, 5′-TCCACCACCCTGTTGCTGTA-3′. Each RT–PCR reaction was performed in duplicate wells using a Taq Man One-step cyber RT–PCR Kit (Takara Biotechnology, Tokyo, Japan). RT reactions were performed at 42 °C for 15 min and 95 °C for 2 min. PCR was performed for 40 cycles at 95 °C for 15 s and at 60 °C for 20 s. GAPDH expression was also analyzed by real-time RT-PCR as a control under the same conditions as for OPN. The relative expression of OPN mRNA was normalized against GAPDH.

Statistical analysis

Results are presented as mean ± s.e.m. Differences between four groups were examined for statistical significance using one-way analysis of variance followed by a modified *t*-test with Bonferroni correction using the StatView program (Abacus Concepts, Berkeley, CA, USA). The Pearson single correlation coefficient was used to correlate OPN mRNA expression with macrophage infiltration and tubulointerstitial injury. A *P*-value less than 0.05 was considered statistically significant.

DISCLOSURE

All the authors declared no competing interests.

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